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Simultaneous determination of azilsartan and chlorthalidone in rat and human plasma by liquid chromatography-electrospray tandem mass spectrometry

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ABSTRACT

Azilsartan medoxomil (AZM), an ester prodrug of azilsartan (AZ), and chlorthalidone (CLT) have recently been approved as a combination therapy for the management of hypertension. This is the first report which described a selective and sensitive method for the simultaneous quantification of AZ and CLT in rat and human plasma using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). AZ and CLT were extracted from plasma by liquid-liquid extraction technique and separated on a C_{18} reverse phase column using ammonium acetate (10 mM, pH 4)-mixture of methanol and acetonitrile (8:92, v/v) as a mobile phase at a flow rate of 0.7 mL/min. Detection was performed by electrospray ionization (ESI) operated in negative multiple reaction monitoring (MRM) mode. The lower limit of quantitation (LLOQ) of this method was 1 ng/mL and the calibration curves were linear ($r^2 \geq 0.995$) over the concentration range of 1–4000 ng/mL for both the analytes. The intra- and inter-day precision and accuracy were well within the acceptable limits. The mean extraction recoveries were found to be about 80% and no matrix effect was observed. AZ and CLT were found to be stable under all relevant storage conditions. The method was successfully applied to the oral pharmacokinetic study of AZM and CLT in rats. Further, the sensitivity of the method enabled the determination of protein binding of AZ and CLT in human plasma.

1. Introduction

Azilsartan medoxomil (AZM), an ester pro-drug is chemically described as (5-Methyl-2-oxo-1, 3-dioxol-4-yl) methyl 2-ethoxy-1-{[2′-(5-oxo-4, 5-dihydro-1, 2, 4-oxadiazol-3-yl) biphenyl-4-yl], methyl}-1H-benzimidazole-7-carboxylate (Fig. 1a). It is a novel, potent and non-peptide angiotensin II type 1 receptor blocker (ARB) that is superior to other existing ARBs in controlling blood pressure [1]. After oral administration, the pro-drug is not detected in systemic circulation because it is hydrolyzed quickly by aryl esterase predominantly in the alimentary tract and/or plasma during absorption to the active moiety, azilsartan (AZ) (Fig. 1b) [2]. Chlorthalidone (CLT), thiazide-like diuretic, is chemically described as (2-chloro-5-(1-hydroxy-3-oxoiso-indolin-1-yl) benzenesulfonamide) (Fig. 1c). Sodium and water depletion is the basis for antihypertensive effect of CLT [3]. Recently, the US Food and Drug Administration (US-FDA) has approved Edarbyclor® that contains azilsartan medoxomil (AZM) and chlorthalidone (CLT) for the treatment of hypertension. It is available in dosage strengths of 40/12.5 mg and 40/25 mg for AZM/CLT.

From a clinical point of view, the combination of AZM/CLT has been shown to be well-tolerated and more effective compared to other antihypertensive combination therapies [4,5]. In pre-clinical studies, it is reported that AZM/CLT afforded the greatest protective effects in hypertension and renal diseases associated with metabolic syndrome [6]. To date, no literature is available describing the pharmacokinetics (PK) of AZM and CLT given in combination in pre-clinical animal species. It is important to monitor the plasma concentrations of AZ and CLT given in combination to develop the temporal relationship between drug exposure and the therapeutic effect. Hence, it is essential to evaluate the PK of AZM and CLT that would assist further pharmacological studies to ensure its rationale

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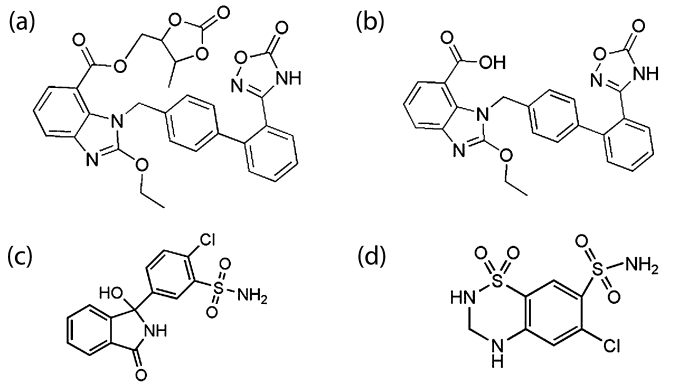

Fig. 1. Chemical structures of (a) AZM, (b) AZ, (c) CLT and (d) IS.

usage with safety and efficacy in several cardiovascular disorders (CVD). A bioanalytical method fit for the purpose of application to PK is required for the simultaneous estimation of AZ and CLT in the biological matrix.

So far, various analytical methods for the quantification of AZM and CLT by reversed phase-high performance liquid chromatography (RP-HPLC) have been reported [1,7,8]. AZM has been determined in human plasma by RP-HPLC method [9], but it could not be utilized for bio-estimation owing to the fact that AZM hydrolyzes rapidly to the active moiety AZ in plasma. Determination of AZM and AZ by HPLC-radiometry has also been reported [2], but suffers from drawbacks of long chromatographic run time, lack of sensitivity, high cost and unsuitable for simultaneous estimation of AZ and CLT in routine sample analysis. Due to the inability of the reported methods to characterize the PK disposition, it is recommended to develop a bioanalytical method that could accommodate the analysis of two individual analytes of interest in plasma. LC-MS/MS has become more definitive technique for simultaneous analysis of multiple compounds in biological matrices due to its inherent selectivity and sensitivity compared to traditional HPLC methods. To the best of our knowledge, LC-MS/MS method has not been reported for the quantification of AZ and CLT in plasma.

The aim of the present work was to develop and validate a simple, selective and sensitive method for the determination of AZ and CLT in rat and human plasma by LC-MS/MS. To demonstrate plasma concentration-time profile, the bioanalytical assay was applied to PK study in male Sprague–Dawley (SD) rats following oral administration of AZM and CLT. In addition, the protein binding of AZ and CLT in human plasma was estimated by in-vitro ultrafiltration technique, thus making it noteworthy to decipher some cues behind the clinical therapeutic drug monitoring in future studies.

2. Materials and methods

2.1. Chemicals and reagents

AZM ($C_{30}H_{23}N_4O_8$, purity ≥ 98%) and CLT ($C_{14}H_{11}ClN_2O_4S$, purity 99%) were gifted from Apotex pharma chem. (Bengaluru, India) and Mylan laboratories (Hyderabad, India), respectively. AZ ($C_{25}H_{20}N_4O_5$, purity ≥ 98%), hydrochlorothiazide (HCT) ($C_7H_8ClN_3O_4S_2$, purity ≥ 98%) which was used as internal standard (IS) (Fig. 1d), HPLC-grade formic acid, acetic acid, ammonium acetate and phosphate buffer saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO). Chromatographic grade-acetonitrile and methanol were supplied by Merck Chemicals (Darmstadt, Germany). Hexane and ethyl-acetate were obtained from Spectrochem Pvt. Ltd (Mumbai, India). Heparin was purchased from Gland Pharma Ltd (Hyderabad, India). Ultrapure water was obtained in-house using a Milli-Q PLUS PF water purifying system (Millipore, Bedford, MA). All other reagents and solvents were of analytical grade and purchased from standard chemical suppliers. Blank, drug-free rat plasma containing heparin as anticoagulant was collected from adult healthy male SD rats. Heparinized drug-free human plasma was obtained from healthy volunteers.

2.2. Liquid chromatography and mass spectrometry conditions

All the bioanalytical procedures were conducted on a liquid chromatographic system (Shimadzu, Kyoto, Japan) equipped to API 4000 QTRAP triple quadrupole mass spectrometer with TurboIon spray interface (Applied Biosystems Sciex, Ontario, Canada). Chromatographic separation was achieved isocratically on a Thermo Syncronis-C_{18} column (100 mm × 4.6 mm i.d., 5 μm) with a C_{18} guard column (Security Guard, Phenomenex, USA) maintained at ambient temperature. The mobile phase consisting of solvent A (10 mM ammonium acetate, pH 4, adjusted using acetic acid) and solvent B (mixture of methanol-acetonitrile, 50:50, v/v) was delivered in the ratio of 8:92, v/v at a flow rate 0.7 mL/min. Autosampler temperature was maintained at 6 ± 2 °C and the injection volume was 10 μL. Rinsing mode was set before and after injection aspiration using acetonitrile: water (50:50, v/v) as a rinsing solvent. The total analysis run time was 2.5 min.

Compound and source dependent parameters were optimized separately for AZ, CLT and IS by infusing neat solutions using Harvard infusion pump (Holliston, MA, USA). Zero air was used as source gas while nitrogen was used as both curtain and collision gas. Multiple reaction monitoring (MRM) was operated at unit resolution for both Q1 and Q3 quadrupoles to identify and quantify deprotonated precursor to product ion transitions in the negative ion mode. The dwell time was set to 200 ms per MRM channel. Data acquisition and integration were controlled by Analyst software 1.6 version (AB SCIEX, Ontario, Canada).

2.3. Preparation of stock and standard solutions

The primary stock solutions of AZ, CLT and IS were prepared in methanol at a concentration of 1 mg/mL. Mixed working solutions of AZ and CLT at various concentration levels were prepared by serial dilution in water-methanol (50:50, v/v; diluent). Calibration standards and quality control (QC) samples were prepared by spiking (2% of total plasma volume) corresponding mixed working solutions to drug-free plasma. The final concentrations in calibration standard samples were 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000 and 4000 ng/mL for AZ and CLT. Quality control (QC) samples were prepared at four different concentration levels as lower limit of quantification (LLOQ – 1 ng/mL), low quality control (LQC – 3 ng/mL), medium quality control (MQC – 400 ng/mL) and high quality control (HQC – 3200 ng/mL). All stock solutions were stored at 2–8 °C.

2.4. Extraction of plasma samples

All the plasma samples were processed by two-step liquid-liquid extraction method. Sequentially, 10 μL of IS working solution (400 ng/mL) and 50 μL of 2% formic acid were spiked to 50 μL of plasma. Then the mixture was extracted with 2.5 mL of *n*-hexane-ethyl acetate (50:50, v/v) by vortex-mixing (Vibrax VXR basic, staufen, Germany) for 5 min and centrifuged at 4000 x g for 10 min (Eppendorf, Hamburg, Germany). The upper clear organic layer was transferred to another set of clean test tubes. The same extraction process was repeated in second step. The combined organic phase was evaporated to dryness under a nitrogen stream at 40 °C, 20 psi (Turbovap®, MA, USA). The dried residue was reconstituted with